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(57) Abstract

The present invention is generally directed to a process for multiplying (cloning) embryos and specifically directed to a process for producing large numbers of identical mammalian embryos by transplanting embryonic stem cell nuclei into enucleated recipient oocytes, enucleated two cell embryos, or replacing the inner cell mass of a mammalian blastocyst with donor mammalian embryonic stem cells. Accordingly, the present invention is also generally directed to a process for cloning live animals by transplanting embryonic stem cell nuclei into enucleated recipient oocytes, enucleated two cell embryos, or replacing the inner cell mass of a mammalian blastocyst with donor mammalian embryonic stem cells.

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TITLE OF THE INVENTION

MAMMALIAN CLONING BY EMBRYONIC STEM CELL (ESC) NUCLEAR TRANSPLANTATION AND ESC

Background of the Invention

Field of the Invention

The present invention is generally directed to a process for multiplying (cloning) embryos and specifically directed to a process for producing large numbers of identical mammalian embryos by introducing embryonic stem cell nuclei into enucleated recipient oocytes, enucleated two cell embryos, or replacing the inner cell mass of a mammalian blastocyst with donor mammalian embryonic stem cells. Accordingly, the present invention is also generally directed to a process for cloning live animals by introducing embryonic stem cell nuclei into enucleated recipient oocytes, enucleated two cell embryos, or replacing the inner cell mass of a mammalian blastocyst with donor mammalian embryonic stem cells.

Description of the Background Art

Embryonic Stem Cells

Embryonic stem cells are pluripotent cells directly derived from the inner cell mass of blastocysts (Evans, M.J. *et al.*, *Nature 292*:154-156 (1981); Martin, G.R., *Proc. Natl. Acad. Sci. U.S.A. 78*:7634-7638 (1981); Magnuson, T. *et al.*, *J. Embryol. Exp. Morph. 81*:211-217 (1982)) (Doetschman, T.C. *et al.*, *Dev. Biol. 127*:224-227 (1988)), from isolated inner cell masses (Tokunaga, T. *et al.*, *Jpn. J. Anim. Reprod. 35*:113-178 (1989)), from disaggregated morulae (Eistetter, H.R., *Dev. Gro. Differ. 31*:275-282 (1989)) or from primordial germ cells (Matsui, Y. *et al.*, *Cell, 70*:841-847 (1992); Resnick, J.L. *et al.*, *Nature, 359*:550-551 (1992)). These cells give rise to the

endodermal, ectodermal, and mesodermal compartments (Doetschman, T.C. *et al.*, *J. Embryol. Exp. Morphol. 87*:27 (1985)).

Embryonic stem cells are able to form permanent cell lines *in vitro*. Mouse embryonic stem cells can be cultured over at least sixty passages and retain, in a majority of cases, a normal karyotype. Embryonic stem cells have been shown to remain in undifferentiated form *in vitro* if maintained on embryonic fibroblast feeder cell layers. In cell suspension, they will begin differentiation, containing elements of glandular, heart, skeletal smooth muscle, nerve, keratin-producing cells, and melanocytes (Doetschman, T.C. *et al.*, *Dev. Biol. 127*:224-227 (1988)).

Embryonic stem cells are the most pluripotent cultured animal cells known. When embryonic stem cells are injected into an intact blastocyst cavity, or under the zona pellucida, at the morula stage embryo, they are capable of contributing to all somatic tissues, including the germ line, in the resulting chimeras (rev'd by Bradley, A., *Curr. Op. Cell. Biol. 2*:1013-1017 (1990); see also Lallemand, Y. *et al.*, *Development 110*:1241-1248 (1990); Bradley, A. *et al.*, *Nature 309*:255-256 (1984); Gossler, A. *et al.*, *Proc. Natl. Acad. Sci. USA 83*:9065-9069 (1986); Robertson, E. *et al.*, *Nature 323*:445-448 (1986)). However, their ability to colonize the various embryonic tissues is not equal. They are able to extensively colonize fetal tissues and extra-embryonic mesoderm, but may be restricted in their capability to contribute to trophectoderm and primitive endoderm derivatives (Beddington, R.S.P. *et al.*, *Development 105*:733-737 (1989); Suemori, H. *et al.*, *Cell Differ. Dev. 29*:181-186 (1990)).

There have been various attempts to obtain embryonic stem cells from pig, sheep, and cattle. These attempts have resulted in the obtaining of "embryonic stem cell-like" cell lines in pig (Strojek, R.M. *et al.*, *Theriogenology 33*:901-914 (1990); Piedrahita, J.A. *et al.*, *Theriogenology 34*:879-901 (1990)), sheep (Piedrahita, J.A. *et al.*, *Theriogenology 34*:879-981 (1990); Notarianni, C. *et al.*, *J. Reprod. Fert. (Suppl.) 43*:255-260 (1991); Karasiewicz, T. *et al.*, *Anim. Sci. Pap. Rep.* (in press 1993)), cattle

(Saito, S. *et al.*, *Roux's Arch. Dev. Biol. 201*:134-141 (1992); Reed, M.A. *et al.*, *Anim. Sci. Pap. Rep.* (in press 1993)), and American mink (Sukoyan, M.A. *et al.*, *Mol. Reprod. Dev. 33*:418-431 (1992)). WO 90/03432 discloses pluripotential embryonic stem cell-like cells derived from porcine and bovine species. This international application describes the production of pluripotential ungulate embryonic stem cells, together with details of the morphology enabling recognition of the cells. However, there is no confirmed evidence that any of these cells are true embryonic stem cells.

Embryonic stem cells can be cultured and manipulated *in vitro* and then returned to the embryonic environment to contribute to all tissues including the germ line (for a review, see Robertson, E.J., *Trends in Genetics 2*:9-13 (1986); Evans, M.J., *Mol. Bio. Med. 6*:557-565 (1989); Johnson, M.P. *et al.*, *Fetal Ther. 4* (Suppl. 1):28-39 (1989); Babinet, C. *et al.*, *Genome 31*:938-949 (1989)). Not only can embryonic stem cells propagated *in vitro* contribute efficiently to the formation of chimeras, including germ line chimeras, but in addition, these cells can be manipulated *in vitro* without losing their capacity to generate germ line chimeras (Robertson, E.J. *et al.*, *Nature 323*:445-447 (1986)).

Embryonic stem cells *in vitro* can be modified by any of the techniques currently known for carrying out manipulation of genetic material. The current methods for gene transfer into cells include transfection, cell fusion, electroporation, microinjection, DNA viruses, and RNA viruses. For a review, see Johnson, M.P. *et al.* (*Fetal Ther. 4* (Suppl. 1):28-39 (1989)). Embryonic stem cells *in vitro* can be modified by any of these techniques and then introduced back into the embryonic environment for expression and subsequent transmission to progeny animals.

Cloning of Identical Mammals

Advanced genetic improvement and selection techniques continue to be sought in the field of animal husbandry. With specific reference to dairy cattle, for example, significant increases in milk production have been made with the wide-scale use of genetically superior sires and artificial insemination. Dairy cows today produce nearly twice as much milk as they did 30 years ago. Further, genetic improvement can be accomplished by the multiplication of superior or genetically manipulated embryos by cloning.

It has now become an accepted practice to transplant embryos in cattle to aid in the production of genetically superior stock. The cloning of embryos together with the ability to transplant the cloned embryos makes it possible to produce multiple genetically identical animals.

Cloned animals have been obtained by the isolation of blastomeres, recombination of isolated blastomeres (chimeric cloning), bisection of embryos at the morula or blastocyst stage, and nuclear transfer. The simplest method to produce identical twins is to bisect morulae or blastocysts and transfer them to synchronous recipients. This technique has been used in cattle (Ozil, J.P. *et al.*, *Vet. Rec. 110*:126-127 (1982); Williams, T.J. *et al.*, *Theriogenology 17*:114 (1982); Ozil, J.P., *Reprod. Fertil. 69*:463-466 (1983); Williams, T.J. *et al.*, *Proceedings of the Annual Conference on AI and Embryo Transfer in Beef Cattle*, Denver, Colorado, January, pp. 45-51 (1983)), sheep (Gatica, R. *et al.*, *Theriogenology 21*:550-560 (1984)), goats (Tsunoda, Y. *et al.*, *Theriogenology 24*:337-343 (1984)), horses (Allan, W.R. *et al.*, *J. Reprod. Fertil. 71*:607-613 (1984); Slade, N.P. *et al.*, *Proceedings of the Congress on Animal Reproduction AI*, vol. 2, no. 241, 3 pp. (1984)), and pigs (Nagashima, H. *et al.*, *Theriogenology 29*:45-495 (1988)). Blastocysts derived from half embryos are smaller than normal blastocysts, but they develop into normal conceptuses.

Various techniques of nuclear transplantation have been used in attempts to produce clones. Success has been reported in preliminary efforts

to adapt nuclear transplant technology to sheep and cattle embryos. Willadsen (*Nature 320*:63-65 (1986)) fused single blastomeres from eight- and sixteen-cell sheep embryos with halves of unfertilized eggs using Sendai virus or electrofusion (Zimmerman, U. *et al.*, *J. Membr. Biol. 67*:165-182 (1982).) Robl *et al.* (*Theriogenology 25*:189 (Abstr.) (1986) reported the transplantation of pronuclei between cow embryos using a method based on the procedure developed by McGrath *et al.* (*Science 220*:1300-1302 (1983)). A karyoplast containing the nucleus was fused with the enucleated embryo by electrofusion. Recently, Bondioli *et al.* (*Theriogenology 33*:165-174 (1990)) reported the birth of seven calves from a single donor embryo using this technique.

The role of whole body and germ cell chimeras in livestock production has been reviewed by Markert (*Proceedings of the Tenth International Congress on Animal Reproduction AI*, vol. 2, pp. 13-19 (1984)). Chimeras may have some use for introducing foreign genes into livestock. In mice, the use of embryonic stem cells to produce transgenic animals by gene targeting has been reported (Capecchi, M.R., *Trends in Genetics 5*:70-76 (1989)). Genes are targeted to embryonic stem cells, which are then incorporated into chimeras. These produce offspring carrying the transfected gene. Mating of chimeric animals may then lead to progeny homozygous for the transgene.

Embryonic stem cells have been used to study *in vivo* events in embryo chimeras. The most commonly used method is the injection of several embryonic stem cells into the blastocoel cavity of intact blastocysts (Bradley, A. *et al.*, *Nature 309*:255-256 (1984)). An alternative method for germ line chimera production involves sandwiching a clump of embryonic stem cells between two eight-cell embryos (Bradley, A. *et al.*, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, ed. Robertson, E.J. (IRL, Oxford, U.K.), pp. 113-151 (1987); Nagy, A. *et al.*, *Development 110*:815-821 (1990)). Both methods result in germ line transmission at high frequency. Recently, an alternative method for the production of embryonic stem cell-embryo chimeras with high levels of chimerism in most, if not all tissues, including the germ line, involves the co-culture of embryonic stem cells with

eight-cell embryos, and follows the same principles as those described for the aggregation of pre-implantation embryos (Tarkowski, A.K., *Nature 190*:857 (1961); Mintz, B., *J. Exp. Zool. 157*:273-292 (1964)) or the sandwiching of clumps of mouse embryonal carcinoma cells (Stewart, C.L., *J. Embryol. Exp. Morphol. 67*:167 (1982) or embryonic stem cells (Nagy, A. *et al.*, *Development 110*:815-821 (1990)) between pre-implantation embryos.

The various procedures to obtain clonal animals have been reviewed (Fehilly, C.B. *et al.*, in *Oxford Reviews of Reproductive Biology*, ed. Clarke, J.R., pp. 379-413, Clarendon Press, Oxford (1986); Modlinski, J.A. *et al.*, in *Future Aspects in Human In Vivo Fertilization*, eds. Feichtinger, W. *et al.*, Springer Verlag, Berlin, Heidleberg, pp. 225-231 (1987); Willadsden, S.M., in *Future Aspects in Human In Vivo Fertilization*, eds. Feichtinger, W. *et al.*, Springer Verlag, Berlin, Heidleberg, pp. 232-237 (1987); Modlinski, J.A., *Biotechnologia 1*:4-13 (1990); Smorag, Z. *et al.*, *Biotechnologia 1*:20-34 (1990)).

However, in spite of the availability of these techniques, only the splitting method of older embryos is commonly used. While other techniques so far have produced only a limited number of cloned animals, this method has resulted in the production of hundreds of twins. This technique, however, drastically limits the number of cloned animals that may be produced and restricts the genetic engineering of the genomes of cloned animals.

Nuclear Transfer

Nuclear transfer was first accomplished in *Amoeba sphaeronucleus* in 1939 by Comandon and de Fonbrune (*Soc. Biol. 130*:744 (1939)). This was followed in 1952 by successful nuclear transfer in *Rana pipiens* by Briggs and King (*Zoology 38*:455-463 (1952)). The procedure for successful nuclear transfers, according to Briggs and King (*supra*) included the following:

(1) the activation of a recipient oocyte;

(2) enucleation, the process of removing or inactivating the chromosomes from the recipient oocyte; and

(3) transfer of a whole lysed blastomere (a cell resulting from embryo cleavage prior to gastrulation), with a nucleus, from a blastula or early gastrula stage embryo back to the enucleated oocyte.

Elsdale *et al.* (*J. Embryol. Exp. Morph.* 8(4):437-444 (1960)), utilized ultraviolet irradiation to, in one step, inactivate the egg pronucleus and activate the unfertilized oocyte. In the axolotl, activation was reported by electrical shock with chromosomes of the egg nucleus being eliminated by ultraviolet irradiation (Briggs, R. *et al.*, *Develop. Biol. 10*:233 (1964)). Transfer of a whole lysed blastomere containing a nucleus into the enucleated oocyte via a small bore micropipette was the common method of nuclear transfer for all these techniques.

Two techniques have been used for nuclear transfer in the mouse. The surgical method of nuclear transfer was developed by Modlinski, J.A., *Nature 273*:466-467 (1978). This was also the first paper concerning nuclear transfer in mammals (see Prather, R.S., in *Molecular Biology of Fertilization*, ed. H. Schatten *et al.*, Academic Press, 1989, pp. 323-340. Pages 325 and 326 contain references and a description of surgical methods for plasma membrane penetration).

Illmensee and Hoppe used a totally surgical method in which a micropipette was inserted through the plasma membrane and into the cytoplasm of a pronuclear stage embryo for nuclear removal and insertion (Illmensee, K. *et al.*, *Cell 23*:9 (1981)). McGrath *et al.* reported a nondisruptive method of transplanting nuclei (McGrath, J. *et al.*, *Science 220*:1300 (1983)). Nuclei were removed as membrane bounded pronuclear karyoplasts without penetrating the plasma membrane of the embryo. The nucleus was inserted into a recipient cell by cell fusion, using Sendai virus as the fusigenic agent. A small volume of Sendai virus suspension was aspirated after removal of the donor nucleus and the virus suspension and the pronuclear

karyoplasts were injected sequentially into the perivitelline space of the recipient embryo. At best, the microsurgical method of Illmensee *et al.* (*supra*) was about 30-40% efficient, whereas the nondisruptive method of McGrath *et al.* (*supra*) was greater than 90% efficient.

It was later reported that blastocyst state embryos and mice were produced by transferring nuclei into enucleated pronuclear zygotes only when the donor cell stage was also pronuclear or at a very early two-cell stage (McGrath, J. *et al.*, *Science 226*:1317-1319 (1984); Surani, M.A.H. *et al.*, *Cell 45*:127-136 (1986); Robl, J.M. *et al.*, *Biol. Reprod. 34*:733-739 (1986)).

Recently, a method for nuclear transplantation in sheep was reported (Willadsen, S.M., *Nature 320*:63-65 (1986)) which describes the use of dielectrophoresis for activation and fusion, and the use of metaphase II oocytes as recipients. These experiments resulted in the birth of cloned lambs.

Summary of the Invention

It is generally an object of the present invention to provide a useful process for improving livestock through cloning genetically superior animals or through genetic engineering of the genome and subsequent cloning of animals with this altered genome.

It is thus an object of the present invention to provide a useful process for producing large or unlimited numbers of cloned animals.

It is a further object of the present invention to provide a useful process for producing large or unlimited numbers of cloned mammals.

It is a further object of the present invention to provide a useful process for producing large or unlimited numbers of cloned mammalian embryos.

It is a further object of the present invention to produce large or unlimited numbers of cloned genetically engineered animals.

It is another object of the present invention to produce large or unlimited numbers of cloned genetically engineered mammals.

It is another object of the present invention to produce large or unlimited numbers of cloned genetically engineered mammalian embryos.

It is a further object of the invention to provide a process for cloning identical animals with cellular and/or nuclear material having a normal developmental capacity.

It is a further object of the invention to provide a process for cloning identical animals with cellular and/or nuclear material having a normal karyotype.

It is a further object of the invention to provide a process for cloning animals wherein the donor genetic material is capable of genetic manipulation.

It is a further object of the present invention to provide a process for the formation of transgenic animals.

These and other objects will become more apparent from the following detailed description and appended claims, as well as the exemplary material provided herein.

Accordingly, the present invention provides a process wherein embryonic stem cells are used as cellular or nuclear donors to produce large or unlimited numbers of genetically identical animals and/or embryos, especially of mammalian origin.

In one embodiment of the invention, embryonic stem cell nuclei are introduced into enucleated oocytes.

In another embodiment of the invention, embryonic stem cell nuclei are introduced into blastomeres of enucleated two celled embryos.

In another embodiment of the invention, whole embryonic stem cells are introduced into a recipient blastocyst from which the inner cell mass has been removed.

Brief Description of the Figures

Figure 1: ***Fusion of cytoplasts carrying embryonic stem cell nuclei with enucleated oocytes and two celled embryos ("indirect fusion")***

Unfertilized zona-free oocytes are divided mechanically into several fragments, or small cytoplasts are removed by micropipette from zona-intact oocytes. Each cytoplast is brought into contact with two to five embryonic stem cells. Such aggregates are then subjected to fusion with PEG, and cytoplasts that have fused with a single embryonic stem cell are injected into enucleated oocytes and two celled embryos. Following microinjection, the oocytes or two celled embryos are exposed to an electric field.

Figure 2A: ***Methods of preparation of inner cell mass-free blastocysts***

A small opening in the zona pellucida (above the inner cell mass) is made either by partial zona dissection using a fine glass needle, or by treatment by acidified Tyrode's solution (step 1). The manipulated blastocysts are cultured in M16 medium at 37°C for six to 12 hours (step 2). The cultured blastocysts undergo expansion, and a small vesticle, usually containing the whole inner cell mass, is extruded through the opening in the zona. The extruded inner cell mass is cut off by a glass needle or surgical blade (step 3).

Figure 2B: ***Methods of preparation of inner cell mass-free blastocysts***

Step 1. The zona pellucida and polor trophectoderm (at the top of the inner cell mass) is punctured by an injection pipette.

Step 2. A fragment of the inner cell mass is sucked into the pipette.

Step 3. The blastocyst is released from the holding pipette, turned over, and sucked again into the holding pipette exactly where the inner cell mass fragment was previously withdrawn.

Step 4. By increasing the negative pressure in the holding pipette, the inner cell mass is subsequently removed from within the blastocyst and cut off by a glass needle or surgical blade.

Step 5. The inner cell mass-free blastocysts are ready for injection (if they have not collapsed) or are cultured for a couple of hours in order to re-expand (if collapsed).

Description of the Preferred Embodiments

Grown *in vitro*, embryonic stem cells provide an unlimited source of theoretically identical cells. Their pluripotency, their ability to respond and to follow normal developmental signals, and their uniform genetic makeup, confer on these cells tremendous value for cloning by nuclear transfer or other techniques. Embryonic stem cells thus provide a route for the generation of transgenic animals, a route which has a number of important advantages compared with more conventional techniques, such as zygote injection and viral infection (Wagner, T.J. *et al.*, in *Experimental Approaches to Embryonic Development*, J. Rossant, *et al.*, eds., Cambridge University Press (1986)), for introducing new genetic material into such animals.

The gene of interest can be introduced and its integration and expression characterized *in vitro*. The effect of the introduced gene on the embryonic stem cell growth can be studied *in vitro*. Then, the characterized embryonic stem cells having a novel introduced gene can be efficiently introduced into embryos by blastocyst injection or embryo aggregation and the consequences of the introduced gene on the development of the resulting transgenic chimeras monitored during pre- or post-natal life. Further, the site in the embryonic stem cell genome at which the introduced gene integrates can

be manipulated, leaving the way open for gene targeting and gene replacement (Thomas, K.R. *et al.*, *Cell 51*:503-512 (1987)).

Accordingly, the present invention is based on a process designed by the inventors wherein cloned animals are produced from embryonic stem cells. The inventors have discovered that when embryonic stem cell nuclei are introduced into enucleated oocytes or two celled embryos, or whole embryonic stem cells introduced into blastocysts from which the inner cell mass has been removed, they can promote fetal development. Thus, the inventors have found a process to utilize all of the advantages and desirable characteristics of embryonic stem cells, discussed above, for the production of cloned animals.

The invention is based on the inventors' consideration that embryonic stem cells can be created from any genetically superior animal or from an animal with specific desirable genetic traits, and passaged *in vitro* to produce theoretically unlimited numbers of cells as a donor source for the formation of cloned animals. These cloned animals may have the same genetic makeup as the donor embryonic stem cell or may contain a genome which has been developed by manipulation of the embryonic stem cell prior to cloning and subsequent development.

Accordingly, one embodiment of the invention is directed to a method of producing cloned animals comprising introducing an embryonic stem cell from any desired animal of any animal species into a blastocyst of said animal species, from which blastocyst the inner cell mass has been removed. The stem cell may or may not be transgenic.

For this embodiment of the invention, the blastocysts may be collected by methods known in the art following mating of the desired animals. Methods of superovulation, mating, and embryo collection, are commonly known in the art and are described in several basic laboratory manuals. The most recent one is *Mammalian Development: A Practical Approach*, ed. M. Monk, IRT Press, Oxford, Washington, D.C. (1987). The inner cell mass is removed from the blastocyst by methods described herein or known in the art. The methods of inner cell mass removal and production of inner cell mass-free

blastocysts, other than those described herein, can be found, for example, in: Gardner, H.L., *Advances in Biosciences 6*:279-296 (1971); Gardner, R.L. *et al.*, *J. Embryol. Exp. Morph. 28*:279-312 (1972); and Gardner, R.L., in *Methods in Mammalian Reproduction*, ed. J.C. Daniel, Academic Press (1978).

In preferred embodiments of the present invention, the inner cell mass is removed by creating a small opening in the zona pellucida, allowing the blastocyst to expand, allowing the extrusion, through the opening, of a small vesicle, usually containing the whole inner cell mass, and the removal of the extruded inner cell mass from the blastocyst. In another preferred embodiment of the invention, the zona pellucida and polar trophectoderm are punctured by an injection pipette, and a fragment of the inner cell mass is pulled outside the zona pellucida by suction into the pipette. The blastocysts should not be too small (i.e., they should have a well developed cavity), not collapsed, and still within the zona. In the method described in Figure 2A, the whole polar trophectoderm is also removed.

In highly preferred embodiments, mouse embryos are removed at the four to eight cell stage, put in M16 medium, and cultured at 40°C for about three days. The result is a trophoblastic vesicle devoid of any inner cell membrane because the high temperature prevents the inner cell membrane from forming. The high temperature treatment can be applied to other animals at the comparable developmental stage, although the exact temperature may not be 40°C. The optimal temperature can be determined by empirical observation.

The injection of embryonic stem cells into blastocysts may be accomplished by methods well-known in the art. For example, for the present invention, embryonic stem cells are introduced into blastocysts by the method of Bradley (in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (ed. E.J. Robertson), pp. 113-151, IRL Press, Oxford, Washington,

D.C. (1987)). The blastocysts with the replaced inner cell mass are then transferred into oviducts of pseudopregnant females according to methods well-known in the art.

In preferred embodiments of the invention, the inner cell mass is removed from blastocysts when the inner cell mass is clearly defined, i.e., forms a discrete mass.

In further preferred embodiments of the invention, more than one embryonic stem cell is introduced into the blastocyst. Normally, in mouse, the inner cell mass contains around 10-15 cells. In a specific disclosed embodiment of the present invention, ten cells are injected into the mouse blastocyst.

In the mouse mid-stage blastocyst, approximately one-third of the total cell number is located in the inner cell mass. (The average cell number in mid-stage mouse blastocyst is about 40-60.) In other species (rabbit, cow, sheep, *et cetera*), around one-third of the cells also probably make up the inner cell mass in relatively early blastocysts. In these species, the trophoblast develops rapidly, and before implantation, is composed of a much higher number of cells than in the mouse. Therefore, if other animal blastocysts are used, around one-third of the total number of cells in a normal blastocyst is transferred or the equivalent of the number of cells in the inner cell mass of that animal species.

By the term "pluripotential cell" or "embryonic stem cell" is intended those cells that retain the developmental potential to differentiate into all somatic and germ cell lineages. The embryonic stem cells or pluripotential cells of the present invention include, but are not limited to, cells derived from humans, mice, sheep, pigs, cattle, and goats.

In another embodiment of the invention, a method is provided for producing cloned animals comprising introducing an embryonic stem cell nucleus from an animal species into an oocyte of said animal species, from which oocyte the nucleus has been removed.

Oocytes are flushed from the oviducts of females that have been induced to ovulate or spontaneously ovulate according to methods well-known in the art.

The embryonic stem cell nucleus may be introduced into the oocyte by methods standard in the art. For example, see Czolowska, R. *et al.*, *J. Cell. Sci. 69*:19-34 (1984) -- PEG fusion; McGrath, J. *et al.*, *Science 202*:56-58 (1984) -- Sendai virus fusion; Willadsen, S.M., *Nature 320*:63-66 ( ) -- Sendai virus fusion and electrofusion; Stice, S.L. *et al.*, *Biol. Reprod. 39*:657-664 ( ) -- electrofusion. For example, it may be introduced into the perivitelline space (transferred under the zona pellucida). In a preferred embodiment of the invention, mid metaphase II oocytes are used as the recipient cell.

In a specific disclosed embodiment of the present invention, nuclear transfer is accomplished by indirect fusion involving polyethylene glycol (PEG) fusion of embryonic stem cells and oocyte fragments or cytoplasts and subsequent fusion of single embryonic stem cells and an oocyte in an electric field.

Unfertilized oocytes are divided mechanically into several fragments (zona-free oocytes) or small cytoplasts are formed from zona-intact oocytes. The cytoplasts or fragments are then exposed to embryonic stem cells to form aggregates. Aggregates are then treated for PEG fusion according to methods known in the art. For example, see Czolowska, R. *et al.*, *J. Cell. Sci. 69*:19-34 (1984). Cytoplasts or fragments fused with a single embryonic stem cell are then injected under the zona pellucida of oocytes. Following injection, the oocytes are exposed to an electric field according to methods known in the art.

In a further preferred embodiment of the invention, nuclear introduction is by direct fusion in an electric field. In this embodiment, oocytes with a single embryonic stem cell introduced under the zona pellucida are exposed to an electric field treatment (electrofusion) according to methods known in the art. Fused oocytes containing the embryonic stem cell nuclei are

then activated prior to implantation into a pseudopregnant female. In a preferred embodiment, activation is with 8% ethanol. The method of ethanol activation is described by Cuthbertson, K.S.R., *J. Exp. Zool.* *226*:331-314 (1983).

By the term "oocyte," for the purposes of the present invention, as used herein for the recipient cell, is intended a cell which develops from an oogonium and, following meiosis, becomes a mature ovum. Not all oocytes are equally optimal cells for efficient donation of an embryonic stem cell in the mouse. For purposes of the present invention, mid metaphase II stage oocytes have been found to be optimal. Mature metaphase II oocytes may be collected surgically from either non-superovulated or superovulated mammals at a predetermined time past the onset of estrus or past an injection of human chorionic gonadotropin (hCG) or similar hormone. Methods of superovulation in domestic animals are routine in the art. Alternatively, immature oocytes may be removed by aspiration from ovarian follicles obtained from slaughtered animals and then may be matured *in vitro* by appropriate hormonal treatment and culturing.

In another embodiment of the invention, a method is provided for producing cloned animals comprising introducing an embryonic stem cell nucleus from an animal species into a two-celled embryo of said animal species, from which two-celled embryo the nuclei of both blastomeres have been removed.

Embryos at the two-celled stage are collected from females by flushing the oviduct following ovulation or superovulation (which may be by hCG injection). In a preferred embodiment of the invention, embryonic stem cells are introduced into one of the blastomeres of the two-celled embryo under the zona pellucida. In a specific disclosed embodiment, cytoplasts or fragments of oocytes that have been fused with a single embryonic stem cell as described above are injected under the zona pellucida. Following microinjection, the

embryo is exposed to an electric field as described above for "indirect fusion." For *in vivo* development, embryos are then transplanted into the oviducts of pseudopregnant females.

By the term "two-celled embryo," for the purposes of the present invention, is intended an embryo after the first post-fertilization cleavage division in which the daughter cells have both completed mitosis, have separate nuclei with nuclear membranes, and have complete individual outer plasma membranes.

In preferred embodiments of the invention, embryonic stem cells at low passage number are introduced into the blastocyst, or provide nuclei for transfer into the embryo or oocyte. The inner cell mass (10-15 cells in mouse) from a blastocyst is introduced into primary culture. After cell divisions leading to the formation of several million cells, the cells are then frozen and stored according to standard procedures (e.g., 7.5% DMSO, packed in styrofoam, allowed to freeze in a mechanical freezer at a rate of around 1°C/minute, and then transferred to liquid nitrogen storage). Aliquots of the frozen cells are then used to re-seed a fresh culture, and cells grown in this first passage culture are then used as the donors for the blastocysts.

In preferred embodiments, the cytoplasts are exposed to two to five stem cells. Since cells have a tendency to disaggregate from the cytoplast, initial incubation with several cells is preferred.

In further preferred embodiments, after injection of embryonic stem cells under the zona pellucida, the oocytes or two-cell embryos are placed in hypotonic medium (50% M2) for around 15 minutes prior to electrofusion. This creates and expands the areas of contact between the embryonic stem cell and the recipient oocyte or embryo. After enucleation, the volume of oocyte/two-cell embryo is diminished, and the perivitelline space becomes larger. Thus, in some cases, the donor embryonic stem cell, floating freely in the perivitelline space, does not have contact with the egg cell membrane. Therefore, areas of contact are preferably expanded as herein described. After

fusion (of the stem cell plasma membrane and recipient cell plasma membrane), the donor nucleus is released into the recipient cytoplasm.

In preferred embodiments of the fusion procedure, a double pulse is used for fusing embryonic stem cells with oocytes or 2-cell embryos. Two 60 V pulses of 30 μseconds apart are administered. Immediately after fusion, the embryos/oocytes are transferred to 37°C.

In further preferred embodiments, a 0.3 M glucose solution is used as the electrofusion buffer.

In further embodiments of the invention, the embryonic stem cells are genetically engineered prior to introduction into the blastocyst, oocyte or embryo. Genetic manipulation can be accomplished by any of the procedures known in the art; for example, by recombinant retrovirus infection, which retrovirus contains genetic material which is desired to introduce into the embryonic stem cell, by transfection procedures, by electroporation procedures, by microinjection, and the like. For reviews of the various methods of genetically engineering embryonic stem cells, see Robertson, E.J., *Biol. Reprod. 44*:238-248 (1991); Wilmut, I. *et al.*, *J. Reprod. Fert. Suppl. 45*:157-173 (1992); Babinet, C. *et al.*, *Genome 83*:938-949 (1989); Evans, M.J., *Mol. Bio. Med. 6*:557-565 (1989); Bradley, A. *et al.*, in *Post-Implantation Development in the Mouse*, Wiley, Chichester (*CIBA Foundation Symposium 165*), pp. 256-276 (1992); Papaioannou, V.E., *Int. J. Dev. Biol. 37*:33-37 (1992)). Examples of desirable genes to be introduced into the embryonic stem cells include, but are not limited to, the genes that control the number of ovulations, litter size, seasonality, prenatal survival, and the determination of sex.

By the term "animal" is intended any living creature that contains cells in which the methods of the present invention can be practiced. Foremost among such animals are animals valuable for the livestock industry; however,

the invention is not intended to be so limiting, it being within the contemplation of the present invention to apply the methods of the present invention to any and all animals for which clones are desirable.

Having now described the invention in general terms, the same will be further described by reference to certain examples that are provided herein and which are not intended to be limiting unless otherwise specified.

Example 1

Removal Of Inner Cell Mass And Replacement With Embryonic Stem Cells

Materials and Methods

1. Preparation of Isolated Inner Cell Mass

Embryonic stem cells were prepared essentially by the method of Robertson described in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (ed. E.J. Robertson) IRL Press, Oxford, Wasington, D.C. (1987), Chapter 4, "Embryo-Derived Stem Cells, specifically pp. 71-112," except that the feeder layer is derived from C578L/6J 12-14 day fetuses, and delayed blastocysts were not used as a source of starting materials.

Inner cell masses were obtained from early four-day blastocysts originating from spontaneously ovulating F1 (C57/BL x CBA/H or C3H x C57/BL) female mice mated either with C57/BL or F1 (C57/BL x CBA/H) males. Isolation of the inner cell mass was performed by treatment with A 23187 calcium ionophore (Sigma) according to the method described by Surani *et al.* (*J. Embryol. Exp. Morph. 45*:237-247 (1978)). The trophectoderm-free inner cell masses were either directly used for experiments or were placed into Ca^{++} and Mg^{++} free M2 medium (Sigma) for 30-40 minutes and then dissociated into two to three fragments. Prior to

microinjection both whole inner cell masses and inner cell mass fragments were incubated in M2 medium containing 1 μg/ml of Cytochalasin D (CD).

2. Preparation of Recipient Inner Cell Mass-Free Blastocysts

Recipient blastocysts were obtained from females of randomly bred Swiss Albino mice, from the inbred BALB/cByJ strain and from the albino standard F1 hybrid strain CAF1/J. In the majority of experiments, spontaneously ovulated females mated with Swiss albino or BALB/cByJ males were used. Blastocysts were collected 3.5 day after mating. After flushing, blastocysts were placed in M2 medium and the inner cell mass was removed using two different methods (Fig. 2A and 2B). In the first method (Figure 2A), a small opening in the zona was made either by treatment with acidified Tyrode's solution (zona drilling) or by partial zona dissection similar to that described by Malter and Cohen (*Gamete Res. 24*:67-80 (1989)). Blastocysts were placed in M16 medium (Sigma) (Whittingham, D.G., *J. Reprod. Fertil. (Suppl) 14*:7-21 (1971)) in 5% CO_2 in air at 37°C. In the following 6-12 hours, the expanding blastocysts extruded a small vesicle through the opening in the zona, usually containing the whole inner cell mass. The extruded inner cell mass was cut off by a glass needle or surgical blade (No. 21). Usually after cutting, the blastocysts did not collapse and were ready for immediate injection. Those which collapsed were cultured in M16 medium until they re-expanded. In some cases, the blastocysts with the extruded inner cell mass were injected with embryonic stem cells prior to removing the inner cell mass.

In the second method (Figure 2B), the zona pellucida and polar trophectoderm, at the top of the inner cell mass, were punctured by an injection pipette and a fragment of the inner cell mass was sucked gently into the pipette and pulled outside the zona pellucida. Then the blastocyst was released from the holding pipette, turned over and sucked into the holding pipette exactly where the inner cell mass fragment was previously withdrawn.

By increasing the negative pressure in the holding pipette, the inner cell mass was subsequently removed from the blastocyst and cut off as described previously.

3. Injection of Inner Cell Masses and Embryonic Stem Cells

Cells were injected into blastocysts using the method described by Babinet *et al.* for inner cell masses (*J. Embryol. Exp. Morph. 60*:429-440 (1980)) and Bradley, A. for embryonic stem cells (in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, ed. Robertson, E.J., pp. 113-151, IRL Press, Oxford, Washington, D.C. (1987)). Whole inner cell masses and inner cell mass fragments were kept in separate drops of M2 plus CD. Blastocysts and embryonic stem cells were kept in CD free M2 medium.

4. Development In Vivo

After the manipulations, the blastocysts were kept in M2 medium for 1-2 hours in order to allow them to re-expand and then were transferred into oviducts of pseudopregnant CD1 or Swiss Albino females mated with vasectomized Swiss albino or BALB/cByJ males, at the first day of pregnancy (day of the vaginal plug). Starting from day 9, a composition of the vaginal smear was checked twice a day. When erythrocytes appeared in a smear, this suggests the beginning of resorption of an embryo. Accordingly, at this time, the recipient female was killed and the embryos removed. After morphological examination the embryos were processed for histology.

5. Control Experiments

Two groups of control experiments were carried out:

A. Between 10-15 embryonic stem cells were injected into intact blastocysts in order to determine if the embryonic stem cell lines growing on

feeder layers or in LIF conditioned medium are capable of colonizing embryonic tissues.

B. Non-injected inner cell mass-free blastocysts were transferred into pseudopregnant recipients to test if the method of inner cell mass removal ensured the elimination of embryonic material.

Results

The majority of the recipient females were killed between the 8th and 9th days. Ten implants were found. These were abnormal egg cylinders which were retarded in development and contained dead cells.

Some of the embryos were able, however, to pass this critical period. Single fetuses were found at days 11 and 13.5. The first one looked apparently normal. The second was slightly retarded in development and large extravasations of blood were visible in the brain region. A small malformation in the facial region was also present. In both conceptuses, the extraembryonic membranes were normal and well developed.

The remaining six conceptuses found between days 11 and 14 were dead and partially resorbed. Histological analysis was impossible.

A single young was born and a second one obtained after a Caesarian section at day 19. The first new-born young, derived from E9 embryonic stem cells, was small, had a large subcutaneous hemorrhage in the posterior part of the body and died after few hours. The second one, obtained from D3 embryonic stem cells, was transferred to a foster mother. It grew very slowly and even with intensive care, died at day 10 after birth.

The replacement of the inner cell mass by either the whole or half of the inner cell mass of different origin resulted in 15.6% (5/32) and 11% (3/27) of live-born young, respectively. No single birth was obtained after transfer of one third of the inner cell mass into the blastocyst cavity. All young developed normally.

D3 embryonic stem cells, either growing on a feeder layer or not, in the presence of LIF , produced chimeras (including germ line chimeras) after injection into intact blastocysts. Also, E9 cells growing in LIF conditioned medium contributed to chimeric mice.

No single birth was obtained after transfer of inner cell mass-free blastocysts. It was found that they were able to induce a decidual reaction, when checked on days 6-7.

Discussion

The results show that embryonic stem cells are able to replace the removed inner cell mass and form the embryo proper of the developing fetus.

During the transition from the early to late blastocyst stage, inner cell mass cells with trophectodermal potential are selectively eliminated by a cytotoxic activity of blastocelic fluid (Pierce *et al., Proc. Natl. Acad. Sci. U.S.A. 86*:3654-3658 (1989); Parchment *et al., Int. J. Dev. Biol. 37*:75-83 (1990); rev'd by Parchment, R.E., *Int. J. Dev. Biol. 37*:75-83 (1993)) and the remaining inner cell mass cells (from which embryonic stem cells are probably derived) are able to differentiate only into embryonic tissues. Therefore, if embryonic stem cells replacing the inner cell mass are able to organize and form a secondary inner cell mass and egg-cylinder, the presence of functional trophectoderm originating from a normal blastocyst should maintain the development of an embryonic stem cell derived embryo. The birth of two young seems to support this hypothesis.

A small number of cells (at least up to 3) is not able to form a functional inner cell mass (Snow, M.H.L., *J. Embryol. Exp. Morph. 35*:81-86 (1976); Markert, C.L. *et al., Science, 202*:56-58 (1978)). One-third of the inner cell mass (around 3-5 cells) cannot sustain development of an embryo (these experiments). Since full term development was obtained (in these studies) after transfer of both whole and half of the inner cell mass into inner

cell mass-free blastocysts, however, this shows that this method of blastocyst reconstruction is effective.

The inability of embryonic stem cell derived neonates to survive, was reported by Nagy *et al.* (*Development 110*:815-821 (1990)) in pups derived from embryonic stem cell-tetraploid aggregates. This indicates that perinatal mortality occurs, in spite of the method of generating embryonic stem cell-derived embryos.

In the experiments herein, embryonic stem cells originated from different mouse strains (D3 - 129/SvJ and E9 - C57Bl/6J). This shows that development to term is also independent of the strain of origin of embryonic stem cell lines. Since in both embryonic stem lines used in the experiments herein, normal diploid cells constituted over 90% of cell population, the detrimental effects of aneuploidy can be excluded, as can the influence of mutation accumulated in a specific cell line. Thus, it might be that during prenatal development, due to a certain incompatibility between the two fetal components of different origin (trophectodermal derivatives and embryonic stem cell derived embryonic tissues), the growing conceptus is nutritionally affected. Nevertheless, a proportion of reconstituted blastocysts resulted in fetuses which were able to undergo full-term development. The possibility of obtaining live-born animals originating from *in vitro* grown embryonic stem cells should not be underestimated, especially for livestock cloning.

Example 2

Embryonic Stem Cell Nuclear Transfer to Oocytes and Two-Celled Embryos

Materials and Methods

I. Embryonic Stem Cell Lines

Two different embryonic stem cell lines were used for these experiments. D3, available from the American Tissue Culture Collection, is a male (XY) line from the 129/SvJ strain of mouse. E9 was derived by the inventors from the C57BL/6J strain using the modification of Robertson described in Example 1 herein.

The embryonic stem cells were cultured either on mitomycin C treated fibroblast cells (from day 12 C57BL/6J mouse fetuses) or in the absence of feeder layer by supplementation with conditioned media containing leukaemia inhibitory factor (Smith, A.G. *et al., Nature 336*:688-690 (1988); Smith, A.G. *et al., J. Reprod. Fert. 84*:619-624 (1988) Williams, R.L. *et al., Nature 336*:648-687 (1988)) (Gibco 5,000 μ/ml).

Embryonic stem cells were grown in 100 mm cell culture Petri dishes pretreated with a 0.1% porcine gelatine solution for at least 1 hour. The medium for the culture of embryonic stem cells was composed of alpha-MEM supplemented with 20% FCS (CC Biologicals, Cleveland, OH, USA), 0.1 mM 2-mercapthoethanol, 10 μg/ml gentamicin and LIF (final concentration of 50,000 U/ml.

For micromanipulation, embryonic stem cells were trypsinized (Robertson, E. *et al., Nature 323*:445-448 (1987)), placed in an equal volume of medium to inactivate the trypsin, and centrifuged at 1500 rpm (Sorvall RT 6000) for 5 minutes. The resulting pellet was resuspended in a small volume of embryonic stem cell culture medium from which a small aliquot was taken

and placed in a drop of M2 medium (Fulton, B.P. *et al.*, *Nature 273*:149-151 (1978)) containing 1 μg/ml of Cytochalasin D (CD) in the micromanipulation chamber.

II. Oocytes and Two-Cell Embryos

Females of the strain Balb/cByJ were used as donors of oocytes and cleaving eggs. Females were kept in a 12 hour day/night regime and induced to ovulate by injection of 5 i.u. PMSG (Sigma) and 5 i.u. hCG (Sigma) given 44-48 hours apart. Oocytes were flushed from the oviducts either 14-14.5 hours (early oocytes) or 16.5-17.5 hours (late oocytes) after hCG injection and treated with 1 mg/ml hyaluronidase (Gibco) to remove the cumulus cells. To obtain 2-cell embryos, the hormonally treated females were mated to Balb/cByJ males. The early, mid and late 2-cell stage embryos were collected 34-44 and 52-53 hours after hCG injection, respectively.

III. Micromanipulation Procedures

Prior to micromanipulation, all oocytes, 2-cell embryos and embryonic stem cells were preincubated for at least 20 min in CD containing M2 medium (1 μg/ml) at room temperature.

1. Enucleation and Embryonic Stem Cell Transfer

Enucleation of the blastomeres at the 2-cell stage was carried out following the method of McGrath, J. *et al.* (*Science 220*:1300-1302 (1983); *Science 226*:1317-1319 (1984)). The unfertilized oocytes were enucleated in a similar manner by removing the metaphase chromosomes arranged in the spindle of the second meiotic division (Czolowska, R. *et al.*, *J. Cell Sci. 84*:129-138 (1986)). Unlike oocytes of other species (cattle: Prather, R.S. *et al.*, *Biol. Reprod. 37*:859-888 (1987); sheep: Smith, L.C. *et al.*, *Biol.*

*Reprod. 40*1027-1053 (1989); rabbit: Stice, S.L. *et al., Biol. Reprod. 39*:657-664 (1988); Modlinski, J.A. *et al., Mol. Reprod. Dev. 28*:361-372 (1991)), in mice, the spindle of the second meiotic division is usually clearly visible under Nomarski's optics and its removal does not create any technical problems.

Immediately after enucleation, embryonic stem cells were introduced (through the same opening in the zona pellucida) into the perivitelline space. In case of oocytes one (or sometimes two) embryonic stem cell was introduced whereas 3-5 cells were transferred under the zona pellucida of 2-cell embryos. For fusion with the two blastomeres of a 2-cell embryo, it was required to have at least two embryonic stem cells. Two-cell embryos are less likely to fuse as frequently with embryonic stem cells as oocytes are. Therefore, in preferred embodiments, it is necessary to increase the fusion efficiency by introducing a higher number of cells.

In a certain number of cases the embryonic stem cells were also introduced under the zona pellucida of non-enucleated oocytes and 2-cell embryos and they were treated by electric field only ("Direct fusion" - see below).

2. Nuclear Transfer

(a) Indirect Fusion (PEG and Electric Field)

In these experiments, only E9 embryonic stem cells were used. Unfertilized zona-free oocytes were divided mechanically into several fragments (Tarkowski, A.K. *et al., Nature 259*:663-665 (1976)). Alternatively, small cytoplasts were removed by micropipette from zona-intact oocytes. Both types of oocytes were preincubated in CD-containing M2 medium. The cytoplasts and embryonic stem cells were placed in BSA-free M2 in wells coated with 1% agar (Sigma). About 5 minutes later, the cytoplasts were transferred to 300-500 μg/ml phytohaemagglutinin (Sigma) in

BSA-free M2 medium and incubated for 5-6 minutes. The cytoplasts were then returned to the well containing embryonic stem cells. Each cytoplast was brought into contact with 2-5 embryonic stem cells. Aggregates were treated for PEG (Fluka, mol.w. 1500) fusion as described by Czolowska, R. *et al.*, *J. Cell Sci.*, *69*:19-34 (1984)). Only cytoplasts which fused with a single embryonic stem cell were selected. These were incubated in CD-containing M2 medium and injected under the zona pellucida of oocytes and 2-cell embryos. Following microinjection, the oocytes/2-cell embryos were exposed to an electric field (see below).

(b) Direct Fusion (Electric Field)

Oocytes and 2-cell embryos containing embryonic stem cells introduced under the zona pellucida were incubated for 30 min in 50% M2 plus CD medium at 37°C in order to enable close contact between the cells to be fused. Before exposure to electric field treatment, they were washed in the same type of solution which was subsequently used for electrofusion.

Three different types of solution have been tested in pilot experiments (results not included in this paper): 0.3 M mannitol (Sigma) supplemented with 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$, 0.3M mannitol, or 0.3M glucose (Sigma) solution supplemented with 10-15 mg/ml BSA (Pentex, Miles Diagnostic). The best results were obtained in glucose plus BSA. Therefore, the overwhelming majority of experiments were carried out using this dielectric solution.

Oocytes/2-cell embryos carrying embryonic stem cells were placed in the electrofusion chamber filled with glucose, between two parallel platinum electrodes located 0.3 mm apart with the contact plane perpendicular to the direction of electric field vector (Kubiak, J.Z. *et al.*, *Exp. Cell Res. 157*:561-566 (1985); Ozil, J.P. *et al.*, *J. Embryol. Exp. Morph.*, *96*:211-228 (1986)). Different fusion parameters were tested in order to establish the best fusion

efficiency and the lowest frequency of lysis of fused cells. Two types of treatment were chosen for further experiments:

(1) Cells were exposed for 10 seconds. to alternating current (A.C., 2.5V, 500kHz) and then submitted to two direct current (D.C.) pulses of 35-45 μ seconds of each at 1.5 kV/cm.

(2) Two D.C. pulses were applied with a duration of 20-30 μ seconds at 2-2.5 kV/cm. A 3312A function generator (Hewlett Packard) served as a source of A.C. and an A310 Accupulser (World Precision Instrument, Sarasota, FL) connected with an A360 Isolation Unit (World Precision instrument, Sarasota, FL) as a source of D.C.

After electric field treatment the oocytes/2-cell embryos were placed in preheated 37°C M2 plus CD medium and checked under Nomarski's optics every 10 minutes. Fusions usually occurred within 5-20 minutes. Occasionally, the non-fused aggregates were treated once again using the same fusion parameters.

3. Activation of oocytes

Oocytes from the experimental and control groups were activated by exposure to a solution of 8% ethyl alcohol in M2 medium for 5 minutes at 37°C (Cuthbertson, K.S.R., *J. Exp. Zool. 226*:331-344 (1983)). Activation of the oocytes receiving the embryonic stem cell nuclei was performed 30-45 min. after electric field treatment.

IV. Control Experiments

1. Efficiency of Activation After Electric Field Treatment

In order to establish if the electric field parameters used for cell fusion acted as an activation agent, unfertilized early and late oocytes (preincubated in CD containing M2 medium) were exposed to the same electric field

parameters as the oocytes in experimental groups and their development was tested *in vivo*.

2. *Efficiency of Activation After Ethanol Treatment*

In these experiments, ethanol was used for activation of oocytes fused with embryonic stem cells. Therefore, the activation efficiency of this agent was tested on intact early and late oocytes preincubated in CD containing M2 medium. The efficiency of activation was evaluated 5-6 hours after ethanol treatment by the number of pronuclear oocytes.

To avoid formation of haploid embryos, which develop poorly, activated oocytes were cultured (prior to transfer to M16 - CD containing medium) for 5-6 hours. This treatment suppresses the extrusion of the second polar body and results in the formation of diploid oocytes. The developmental abilities of activated oocytes were tested after 3-4 days of *in vivo* culture in the oviducts of immature females (approx. 3 weeks old).

3. *Influence of Electric Field Treatment on the Development of Activated Oocytes and 2-Cell Embryos*

In order to determine if the applied electric field can affect development, early and late 2-cell embryos were treated with the same electric field parameters and then transferred into pseudopregnant recipients.

V. *In Vitro and In Vivo Culture*

For *in vitro* development, activated oocytes and 2-cell embryos were cultured in M16 medium at 37°C in 5% CO_2 in air. For *in vivo* development, oocytes/2 cell - embryos were transplanted either to the oviducts of pseudopregnant CD1 recipients mated with vasectomized Balb/cByJ males (for

pre- and postimplantation development) or into ligated oviducts of immature (3-4 week old) CD1 females (for preimplantation development only).

VI. Cytological analysis

The obtained preimplantation embryos derived from reconstituted oocytes and 2-cell embryos were fixed in Heidenhein's fixative, whole-mounted (Tarkowski, A.K. *et al., J. Embryol. Exp. Morph. 18*:155-180 (1976)) and stained with Harris hematoxylin.

Results

1. Fusion of Embryonic Stem Cells with Enucleated Oocytes and 2-Cell Embryos

Oocytes fuse more easily with embryonic stem cells than do 2-cell embryos (40%-90% vs. 15%-35% - Table 1.). Also, early oocytes and embryos fuse better than the late ones. Differences in the fusogenic abilities between oocytes and 2-cell embryos were even more significant since the majority of oocytes was able to fuse when a single embryonic stem cell only was introduced into the perivitelline space, whereas in order to obtain fusions between 2-cell embryos and a single embryonic stem cell, 3-5 cells had to be introduced under the zona pellucida (see Materials and Methods). A slightly higher fusion rate was also obtained (45%, 22 out 48) after using the indirect method of fusion of 2-cell embryos with cytoplasts carrying a single embryonic stem cell nuclei. This method however, provides the controlled introduction of a single nucleus.

2. *Behavior of Embryonic Stem Cell Nuclei in the Cytoplasm of Oocytes and 2-Cell Embryos*

(a) Non-activated oocytes

Embryonic stem cell nuclei underwent premature chromosome condensation (PCC) when introduced to the cytoplasm either of intact or enucleated oocytes. The dispersion of the nuclear envelope and a disappearance of nucleoli were observed 15-30 minutes after fusion. Although no direct comparison was carried out, it appears that PCC often took place earlier in intact oocytes than in enucleated ones. Usually, after approximately 60-75 minutes, PCC was completed and the condensed chromatin was arranged in more or less regular PCC figures.

(b) Activated oocytes

The number of reconstituted early oocytes that were activated after ethanol treatment (i.e. in which prophase nuclei of embryonic stem cell origin were formed) was relatively high (approx. 70-80% of the treated oocytes in some experiments), and similar to that observed in the control group. In early oocytes, usually 16.5-17.5 hours after hCG (at the fusion-activation time), the nuclei grew rapidly during the first 5-6 hours, and reached at least the size of full grown pronuclei at the end of the first cell cycle. In intact oocytes, embryonic stem cell nuclei behave normally by swelling (increasing in size) and stain as do nuclei containing decondensed chromatin. Alternatively, the nuclei may remain in a condensed state (darkly staining). On the other hand, in about one third of the enucleated, activated early oocytes, the introduced embryonic stem cell nuclei swelled enormously reaching, in the most extreme cases, three-fourths of the diameter of an oocyte (i.e. 40% of an oocyte's total volume). Also, about one-third of the enucleated, activated oocytes receiving embryonic stem cell nuclei, extruded "a second polar body" 1.5 hours after

activation, i.e., at the normal time for the fertilized or artificially activated oocytes, if they were cultured in CD free M16 medium. In these "polar bodies" a morphologically normal looking nucleus was formed. In the majority of cases, oocytes containing "overgrown" nuclei were unable to pass the first cleavage division, and, in several cases were also unable to enter even the onset of the first mitosis.

In late oocytes, nuclei also swelled rapidly but only occasionally formed the "gigantic" nuclei observed in early oocytes. Extrusion of "a second polar body" sometimes also occurred. However, fragmentation more frequently was observed in aged, activated oocytes (if they are cultured in CD free M16 medium). Since fragmentation, or rather increased cortical activity leading to the extrusion of several small cytoplasmic fragments, was sometimes also observed in early oocytes, we decided to culture both early and late oocytes in CD containing M16 medium up to 5 hours after activation. Such a treatment prevents fragmentation and extrusion of "a second polar body". While swelling, the introduced embryonic stem cell nuclei moved towards the center of the oocytes (in CD-free medium). However, some late oocytes remained located close to the inner surface of an egg membrane.

A cytological analysis of reconstituted oocytes revealed two different patterns of remodelling of embryonic stem cell nuclei. In approximately three-fourths of the nuclear fusion oocytes, the swelling of embryonic stem cell nuclei was accompanied by decondensation of chromatin and formation of a chromatin net and nucleoli-like structures characteristic of the pronuclear stages. In the remaining one-fourth of reconstituted oocytes, the introduced nuclei were darkly stained (regardless of size, although never exceeding the pronuclear size). The nucleoli were located near the nuclear envelope, which, in these remodelled nuclei, was not smooth (as in the above mentioned group) but more or less crenated.

(c) Two-Cell Embryos

In enucleated early two-cell embryos, embryonic stem cell nuclei either remained unchanged or underwent the first stage of remodeling. In almost half of the enucleated early 2-cell embryos, the embryonic stem cell nuclei swelled and became oval-shaped (rather than round as was observed in nuclear transfer oocytes). They also stained rather darkly. Their size did not exceed the size of half-blastomere nuclei. They were usually about one-third smaller. The cytological analysis of mid-late reconstituted 2-cell embryos was not undertaken. They were immediately transferred to the oviducts and checked either on the next day (immature females - see below) or were left for postimplantation development (pseudopregnant females).

3. Preimplantation Development of Reconstituted Oocytes and 2-Cell embryos

More than half of the reconstituted early oocytes and late 2-cell embryos underwent the first cleavage division. This resulted in the formation of more or less regular 2-cell embryos, or 3-4 cell embryos, depending (in the latter case) on the number of blastomeres that fused with embryonic stem cells. At least half cleaved normally; that is, of the cleaving reconstituted oocytes and 2-cell embryos, approximately 50% looked apparently normal. The others formed an unequal sized one-half or one-fourth blastomere. Cases in which only one blastomere developed normally and the second one fragmented, degenerated, or was without a visible nucleus, or when the developing embryos were composed of extremely different sized blastomeres, were not included in the Table 1.

The majority of late oocytes and early-mid 2-cell embryos did not cleave or fragment. Only about a fourth were able to pass to the next cleavage division. However, almost all that did cleave formed regular 2-, or 3- or 4-cell embryos. To avoid the "2-cell block" to further post-implantation

development, reconstituted oocytes were cultured *in vitro* for 24 hours, in which time they underwent a cleavage division.

A majority of nuclear transfer oocytes, and all of the reconstituted early and mid 2-cell embryos, were transferred to the oviducts of immature recipient females. Their development was checked after 3-4 days. The recovered embryos were mainly in a variety of stages of arrested development after one or two cleavage divisions, were fragmented, degenerated or otherwise appeared abnormal. However, some of the recovered embryos were able to pass at least three cleavage divisions.

Around half of those embryos passing three cleavage divisions deviated from normal, and primarily consisted of (1) embryos with binuclear cells, (2) multicellular embryos with abnormal appearing, different sized blastomere nuclei, (3) embryos which, after staining, were shown to be partially composed of anucleate cytoplasmic fragments, and (4) non-compacted morulae. In spite of these developmental irregularities in many cases, however, 10%, 8% and 18% of reconstituted early-, late oocytes, and early 2-cell- embryos respectively, were able to develop until the late compacted morula or blastocyst stage. (Table 1.)

TABLE 1: TRANSFER OF EMBRYONIC STEM CELL NUCLEI INTO ENUCLEATED MOUSE OOCYTES AND CLEAVAGE EMBRYOS

Type of Experiment	Fusion Rate * (%)	Preimplantation Development		Postimplantation Development	
		One Cleavage *in vitro* or *in vivo* **	Morulae/Blastocysts *in vivo*	Up to Day 10	Beyond Day 10
Early Oocytes 15-16 hrs after hCG	70-90	31/50 (62%)	13/130 (10%)	12/34 (9%)	4/96 (4%)
Late Oocytes 18-22 hrs after hCG	40-50	39/125 (31%)	11/36 (8%)	7/72 (5.5%)	3/131 (2%)
Early-Mid 2-cell embryos 30-45 hrs after hCG	30-35	45/182 (25%)	28/152 (18%)	8/85 (9.7%)	13/146 (9%)
Late 2-cell embryos 50-54 hrs after hCG	15-20	26/45 (58%)	---	---	3/18 (16.5%)

* Numbers represent a mean fusion rate obtained by combining results of experiments using direct and/or indirect fusion techniques

** in oviducts

In the case of morula/blastocyst originating from transfer to 2-cell embryos, the majority developed from a single blastomere and thus were smaller and composed of a lower number of cells than the oocyte transfers. Another difference between the developmental potential of the oocytes and 2-cell embryos was that only 15 of 28 normally developing early transfer embryos reached the blastocyst stage at day 4. The others were at the morula stage and only a few started to cavitate during the 24 hours *in vitro*. But 10 out of 13 and 8 out of 11 blastocysts were derived from reconstituted early and late oocytes respectively.

In at least four blastocysts from early transfer oocytes and five derived from late oocytes, binucleate cells were found among the inner cell mass cells (binucleate cells indicate tetraploid embryos). In such binucleate cells, two diploid nuclei can form, during the next cleavage division, one tetraploid nucleus. Thus, in the developing embryo, some cells will be diploid and some will be tetraploid. In mammals, diploid/tetraploid mosaics do not develop normally.

4. Post implantation development

The development of reconstituted oocytes and 2-cell embryos was checked after transfer to the oviducts of pseudopregnant females a few days after implantation (up to day 10), or after the 10th day. Simultaneous testing of vaginal smears was also done. The composition of the vaginal smear, especially the presence or absence of blood beginning at day 11, was the main indicator of the developing pregnancy.

About 12% (16/130), 5% (10/203), and 9% (21/231) of embryos originating from nuclear transfer to early oocytes, and late oocytes, and early 2-cell embryos respectively, were able to implant. The implantation rate of reconstituted late 2-cell embryos was higher (16%).

The potential for further normal development was then tested. Out of twelve implants from nuclear transfer to early oocytes (7 implants recovered

at day 9 and 5 implants at day 10), two 9-day foetuses and one 10-day foetus were alive. All three were normal in all respects but development was retarded about one day.

Five implants from nuclear transfer to late oocytes were found at day 8 and two were found at day 10. In three of the 8th day implants, no embryonic tissues were found. The two others were apparently normal and were not retarded in development. One of the 10 day conceptuses contained the extraembryonic tissues only. The other was healthy with no visible signs of developmental retardation.

Eight implantation sites originating from early 2-cell embryos receiving embryonic stem nuclei were checked at day 10. All but one contained only degenerated tissue. In the remaining one, a living foetus corresponding to 8 1/2 days of development was found.

Successful development beyond day 10 was found in all groups of reconstituted oocytes/2-cell embryos. At day 13, one normal appearing foetus originating from reconstituted early oocytes was observed. One foetus corresponding to day 11/12 derived from early 2-cell embryos was also observed. Both foetuses originated from E9 cell nuclei. A third 16 day foetus, from nuclear transfer of D3 cell nuclei to a late 2-cell embryo, was also obtained. That foetus looked entirely normal, was fully grown, and had black pigmented eyes indicating the 129/SvJ strain origin of the introduced nucleus.

5. Control experiments

The applied electric field activated only 4.7% (2/42) of early and 10% (5/51) of late oocytes. The ethanol treatment, however, appeared to be a highly effective activation agent. Five-six hours after ethanol activation and culture, 65.6% (40/61) of early and 86.2% (50/58) of late oocytes were activated. After transfer of 32 early and 38 late diploid oocytes into ligated oviducts of immature females, 23 and 22 blastocysts were found, respectively.

The applied electric field does not affect the development of early and late 2-cell embryos. Out of 19 late and 21 early 2-cell embryos which were treated with the same electric field parameters as the experimental embryos and transferred to pseudopregnant recipients, 13 and 15 young were born respectively.

Discussion

Mouse embryonic stem cells are the only known cell type which retain pluripotency *in vitro*. They also appear to be fully totipotent, since it is possible to obtain live born mice originating entirely from embryonic stem cells (Nagy, A. *et al.*, *Development 110*:815-821 (1990)). The question of whether the totipotency of whole embryonic stem cells extends to the cell nucleus was addressed in these studies. In some mammalian species, such as rabbit, sheep, and cattle, the totipotency of isolated blastomeres and their nuclei is equivalent, at least, to the 8-cell stage (for review see: Modlinski, J.A. *et al.*, In *Future Aspects in Human In Vitro Fertilization*, eds. Feichtinger, W. *et al.* Springer Verlag, Berlin, Heidlberg, pp. 225-235 (1987); Willadsen, S.M., *Id.* pp. 232-237; Modlinski, J.A., *Biotechnologia, 1*:4-13 (1990); Prather, R.S., in *The Molecular Biology of Fertilization* (eds. H. Schatten *et al.*, pp. 323-340, Academic Press, Inc. (1989)). However, the ability the of the nucleus to promote full term development extends well beyond this stage (morulae cell nuclei in cattle - Prather, R.S. *et al., Biol. Reprod. 37*:859-888 (1987); inner cell mass cell nuclei in sheep - Smith, L.C. *et al., Biol. Reprod. 40*:1027-1053 (1989)). Prior to these studies, in the mouse, it was still not clear at which stage the embryonic nuclei retain their totipotency and the capability to support development after transfer to recipient cytoplasm. It was commonly considered, that this ability is restricted only to the early stages. Recent observations of Tsunoda *et al., Development 107*:407-412 (1989) and Kono *et al., J. Reprod. Fert. 93*:165-172 (1991)) indicated that mouse inner cell mass cell nuclei were able to support

development, at least to the blastocyst stage. The totipotency of embryonic stem cells and the reported capabilities of some mouse inner cell mass nuclei in promoting development led the inventors to investigate the developmental potential of embryonic stem cell nuclei in various cytoplasmic environments.

Accordingly, the present studies concentrated on: (1) the behavior of embryonic stem cell nuclei in early/late oocytes and early 2-cell embryos; (2) preimplantation development of reconstituted oocytes/2-cell embryos; and (3) postimplantation development of embryonic stem cell nuclear transfer oocytes/2-cell embryos.

1. Behavior of Embryonic Stem Cell Nuclei in Enucleated Oocytes and 2-Cell Embryos

For successful development of reconstituted oocytes the donor nucleus should initially undergo a sequence of changes, referred to as "remodelling" (Czolowska, R. *et al., J. Cell Sci. 69*:19-34 (1984)). The changes result in morphological and metabolic resemblance to a pronucleus. The first indication of remodelling is the swelling of the donor nucleus in amphibians (Gurdon, J.B., *Adv. Morphol. 4*:1-41 (1964); Gurdon, J.B., *J. Embryol. Exp. Morph. 20*:401-414 (1968)); and in mammals (mouse: Czolowska, R. *et al., J. Cell. Sci. 69*:19-34 (1984) rabbit: Stice, S.L. *et al., Biol. Reprod., 39*:657-664 (1988); Modlinski, J.A. *et al., Mol. Reprod. Dev. 28*:361-372 (1991); pig: Prather, R.S. *et al., Biol. Reprod. 41*:414-418 (1989)). This nuclear swelling may be induced by the entry of certain cytoplasmic proteins into the nucleus (Merriam, R.W., *J. Cell Sci. 5*:333-349 (1969)) and a consequence of the exchange of proteins between the recipient cell cytoplasm and the nucleoplasm of the donor nucleus (DiBerardino, M.A. *et al., Exp. Cell. Res. 94*:235-252 (1975), Leonard, R.A. *et al., Dev. Biol. 92*:343-355 (1982).

A swelling of embryonic stem cell nuclei prior to formation of pronucleus-like structures was observed in about two-thirds of early, and in the majority of late nuclear transfer oocytes. This suggests that until 10 hours

after ovulation (22 hours after hCG injection), the oocyte cytoplasm is generally still capable of controlling pronuclear formation and remodelling of foreign nuclei. However, the abnormal swelling of some embryonic stem cell nuclei, prior to the formation of gigantic pronuclei-like structures, indicates that a certain equilibrium in protein exchange, probably present during normal pronuclear growth, is disturbed and, thereby, cytoplasmic control over nuclear remodelling is lost.

Several enucleated early oocytes receiving embryonic stem cell nuclei extruded, after ethanol activation, a second polar body-like structure. These "false" polar bodies were bigger than true polar bodies, but always contained a single, interphase nucleus. Since the applied electric field activated a very low proportion of both early (4%) and late (10%) oocytes, the overwhelming majority of introduced embryonic stem cell nuclei should have undergone premature chromosome condensation before the oocytes were subjected to the ethanol treatment. As was documented by Longo *et al.* (*Dev. Biol. 107*:382-394 (1985)) and by Maro *et al.* (*J. Embryol. Exp. Morph. 81*:211-217 (1984); *J. Cell Biol. 101*:1665-1672 (1985)), chromosomes of free chromatin induce an actin-rich filamentous layer overlaid by a smooth cell membrane in the vicinity of the chromosomes. Szollosi *et al.* (*Eur. J. Cell Biol., 42*:140-151 (1986)) and Soltynska *et al.* (*Biology of the Cell 57*:135-142 (1986)) observed similar cortical changes over the thymocyte chromatin in oocyte-thymocyte hybrids. It appears that in embryonic stem cell-enucleated oocyte hybrids, the oocyte cytoplasm can control the donor nuclei and the denuded embryonic stem cell chromatin can induce the cortical layer which cuts off the second polar body.

The extrusion of a "false" second polar body can result in formation of either haploid or diploid embryos, depending on whether pre-, or postsynthetic nuclei are transferred. This, therefore, can explain the developmental failure of some of the reconstituted oocytes. The development of haploid embryos is usually limited and although some are able to pass the preimplantation period of development, they can provoke, after implantation,

only a decidual reaction (rev'd by Kaufmann, M.H., in *Early Mammalian Development: Parthenogenetic Studies*, London: Cambridge University Press (1983); Ozil, J.P., *Development 109*:117-128 (1991). Extrusion of the second body was more evident with embryonic stem cell nuclear transfer to early oocytes. Late oocytes, soon after fusion and activation, usually started to cleave abnormally, fragmented, or extruded small cytoplasmic fragments. In aging oocytes, the mitotic spindle may become translocated towards the oocyte center. This results in immediate or abnormal cleavages after activation (rev. by Kaufmann, M.H., *Early Mammalian Development: Parthenogenetic Studies*, London, Cambridge University Press (1983). This was not the case in the studies herein, however. No rapid movement was observed immediately after transfer of embryonic stem cell nuclei into late vs. early oocytes. It is more likely that during aging, changes occur in the oocyte membrane and in the cortical layer which make it more fragile and sensitive to activation factors.

Embryonic stem cell nuclei introduced into 2-cell embryos were placed in a foreign cytoplasmic environment. In the early 2-cell embryo, the appearance of a novel group of 68-73 x 10,000 Mr proteins, whose synthesis is inhibited by alpha amanitin, is thought to mark the starting point of transcriptional activation of the embryonic genome (Flach, G. *et al., EMBO J. 1*:681-686 (1982); Bensaude, O. *et al., Nature 305*:331-333 (1983); Bolton, V.N. *et al., J. Embryol. Exp. Morph. 79*:139-163 (1984)). The synthesis of these proteins, designated transcription-requiring complex (TRC), appear to be 2-cell stage specific. They are not detected in one celled embryos and are essentially degraded in 8-cell stage embryos (Conover, J.C. *et al., Dev. Biol. 144*:392-404 (1991)). When nuclei originating from more advanced embryos were transferred to enucleated zygotes, the synthesis of the TRC was observed at the time that corresponded to transcriptional activation. (Howlett, S.K. *et al., Development 101*:915-923 (1987)), and Latham & Schultz, cit. by Conover, J.C. *et al., Dev. Biol. 144*:392-404 (1991)). But this reprogramming was not sufficient to support development beyond one or two

cleavage divisions (Howlett, S.K. *et al., Development 101*:915-923 (1987)). However, when 8-cell stage nuclei were introduced into enucleated 2-cell embryos, they could support preimplantation (Howlett, S.K. *et al., Development 101*:915-923 (1987)), early postimplantation (Robl, J.M. *et al., Biol. Reprod. 34*:733-739 (1986)), and could even produce live-born young (Tsunoda *et al.*, (1987) Kono, T. *et al., Gamete. Res., 24*:375-384 (1989)).

Similar results obtained in the experiments herein (normal development until day 13-16) indicate that in spite of, or maybe due to, the pre-existing initial transcriptional activity, the environment of 2-cell stage cytoplasm seems be more propitious for nuclear reprogramming than the zygote's cytoplasm which may contain unfavorable stage-specific factors (Surani, M.A.H. *et al., Biol. Reprod. 36*:1-16 (1987)).

The nuclear remodelling of embryonic stem cell nuclei introduced into enucleated early 2-cell blastomeres was expressed by their swelling and consequent formation of oval-shaped nuclei, approximately one-third smaller than the size of half blastomere nuclei. In non-enucleated 2-cell embryos, the donor nuclei were not usually able to pass the first steps of morphological remodelling. This suggests that in early 2-cell embryos, a certain pool of proteins (some probably released in to the cytoplasm during the first mitotic division) are still available, whereas in nonenucleated embryos, their acquisition by the donor embryonic stem cell nuclei is suppressed by the resident blastomere nuclei. In enucleated half blastomeres, embryonic stem cell nuclei are not handicapped by the competition for these cytoplasmic components.

Although the behavior of embryonic stem cell nuclei in late 2-cell embryos was not monitored, it is probable that in such embryos that are already entering the M phase, the donor nuclei undergo chromosome condensation and after completion of mitosis begin reprogramming in full synchrony with the recipient blastomere next cell cycle. In the case that G2 nuclei are introduced, this model might be optimal for further development of

reconstituted embryos. Proper synchrony between the nucleus and cytoplasm appears important in the development of reconstituted embryos.

2. & 3. Pre-, and Postimplantation Development of Reconstituted Oocytes and Two-Cell Embryos

Since nuclear transfer to early and late oocytes resulted in a greater number of blastocysts than did nuclear transfer to early 2-cell embryos, it appears that the exposure of the denuded embryonic stem cell chromatin to factors in the oocyte cytoplasm results in more faithful remodelling of embryonic stem cell nuclei. The exposure of denuded chromatin to oocyte cytoplasm is a prerequisite for morphological and functional remodelling of mouse thymocyte nuclei and erythrocyte nuclei (rev. by DiBerardino *et al.*, *Science 224*:946-952 (1984). These nuclei formed a new nuclear envelope (Szollosi, D. *et al., Biology of the Cell, 56*:239-250; *Eur. J. Cell Biol. 42*:140-151 (1986); *J. Cell. Sci., 91*:603-613 (1988)) and initiated the remodelling program in accordance with the recipient cell cycle. A similar situation probably occurs in the late 2-cell nuclear transfer embryos (see above). Also, extrusion of the second polar body in early oocyte-embryonic stem cell hybrids, indicates that the decondensed chromosomes of embryonic stem cell origin were arranged in a regular spindle (a phenomenon rarely reported in oocyte-interphase cell hybrids), following signals transmitted from the oocyte cytoplasm. However, in early 2-cell embryo cytoplasm, the donor embryonic stem cell nuclei did not appear to fully reprogram, as evidenced by limited swelling (this study) and by smaller size and fewer cell numbers in blastocysts derived from 2-cell embryos with 8-cell nuclei (Robl, J.M. *et al., Biol. Reprod. 34*:733-739 (1986)). Although the swelling of embryonic nuclei after fusion with fertilized rabbit (Modlinski, J.A. *et al., Mol. Reprod. Dev. 28*:361-372 (1991) and activated mouse oocytes (unpublished observation from Department of Embryology, University of Warsaw; cited by Szollosi, D. *et al., J. Cell Sci. 91*:603-613 (1988) does not depend on nuclear envelope

breakdown, it is still possible that these nuclei are not able to undergo the full remodelling program.

The wide spectrum of different abnormalities appearing during preimplantation development in both oocyte and 2-cell nuclear transfer embryos clearly reflects difficulties in mutual adjustment of two components in reconstituted nucleocytoplasmic hybrids. Since similar abnormalities were observed after transfer of various embryonic nuclei of different species, they can not be attributed directly to specific properties of embryonic stem cell nuclei. They rather suggest more general mechanisms affecting the reprogramming of embryonic nuclei. Premature compaction, suggesting a control of the donor nucleus over the recipient cytoplasm, was reported both in mouse (Howlett, S.K. *et al., Development 101*:915-925 (1987); Tsunoda, Y. *et al., J. Exp. Zool. 242*:147-151, (1987)) and in rabbit (Modlinski, J.A. *et al., Mol. Biol. Reprod. Dev. 28*:361-372 (1991)). Lack of compaction, especially when the nuclei from post-compaction stage embryos were transferred, was also reported (mouse - these studies; rabbit - Modlinski, J.A. *et al., Mol. Biol. Reprod. Dev. 28*:361-372 (1991)) sheep - Smith, L.C. *et al., Biol. Reprod. 40*:1027-1053 (1989)). Delayed or premature cleavage divisions (Ozil & Modlinski, unpublished; Waksmudzka, pers. inf.; (Modlinski, J.A. *et al., Mol. Biol. Reprod. Dev. 28*:361-372 (1991)) and also these studies) reflect asynchrony in cell cycles between the foreign nucleus and recipient cytoplasm. Although the normality of chromosomes in reconstituted egg cells has never been examined, asynchrony can lead to chromosomal abnormalities that could contribute to decreasing developmental abilities, especially post-implantation.

In some developing embryos, binucleate cells were found, indicating karyokinesis without cytokinesis. A presence of binucleate cells can result in formation of diplo-tetraploid mosaics. Tetraploid embryos (as well as octoploid, Waksmudzka & Modlinski, unpublished) can implant at a high rate but they rarely form embryonic structures (Snow, H.H.L., *J. Embryol. Exp. Morph. 34*:707-721 (1975); *J. Embryol. Exp. Morph. 35*:81-86 (1976);

Tarkowski, A.K. *et al., J. Embryol. Exp. Morph. 41*:47-64 (1977)) and very seldom result in live-born young (mice: Snow, M.H.L., *Nature, 244*:513-515 (1973); Human: Golbus, Y.S. *et al., J. Med. Gen. 13*:329-332 (1976)). Although in 2N/4N chimaeras, tetraploid cells are not usually eliminated during development of embryonic tissues (Lu, T.Y. *et al., Proc. Natl. Acad. Sci. U.S.A. 77*:6012-6016 (1970); Nagy, A. *et al., Development 110*:815-821 (1990), several 2N/4N mosaics die around mid-pregnancy (mouse - Tarkowski, A.K. *et al., J. Embryol. Exp. Morph. 41*:47-64 (1977); rabbit - Ozil, J.P. *et al., J. Embryol Exp. Morph. 96*:211-228 (1986). Occasionally they are able to reach more advanced stages of development (Ozil, J.P. *et al., J. Embryol. Exp. Morph., 96*:211-228 (1986)) or survive until birth (human - Kelly, T.E. *et al., Clin. Gen. 6*:221-224 (1974)). Also, Nagy, A. *et al., Development 110*:815-821 (1990), suggest that the mortality of embryonic stem cell derived embryos may be due to the presence of aneuploid cells in cultured embryonic stem cell populations. However, in the present studies, the karyotypic analysis of D3 and E9 embryonic stem cell lines showed that approximately 90% had a normal diploid karyotype.

Embryonic stem cells are, however, different than the other mammalian embryonic cells hitherto used in nuclear transfer experiments. They are derivatives of the inner cell mass of very late blastocysts. In early blastocysts, the inner cell mass still retains a potential to differentiate into trophectodermal cells (Hogan, B. *et al., J. Embryol. Exp. Morph., 45*:93-105 (1978); *62*:379-394 (1981)). During transition from early to late blastocyst stage, the inner cell mass cells with trophectodermal potential are gradually eliminated by apoptosis, i.e. by a programmed cell death caused by the cytotoxic activity in blastocelic fluid (Pierce, G.B. *et al., Proc. Natl. Acad. Sci. U.S.A., 86*:3654-3658 (1989); Parchment, R.E. *et al., Differentiation, 43*:51-58 (1990); rev'd by Parchment, R.E., *Int. J. Dev. Biol. 37*:75-83 (1993)). Therefore, in late blastocysts, the remaining inner cell mass cells lack the ability to form trophectoderm and are able to differentiate only into embryonic tissues (Papaioannou, V.E. *et al., Nature 258*:70-73 (1975); Mintz, B. *et al., Proc.*

Natl. Acad. Sci. USA 72:3585-3589 (1975)). Moreover, a similar cytotoxic activity was found in the fluid of cystic embryoid bodies of C44 embryonal carcinoma, which preferentially killed embryonal carcinoma cells with trophectodermal potential (Parchment, R.E. *et al., Differentiation, 43*:51-58 (1990)). It is known, indeed, that in chimeras, mouse embryonic stem cells can contribute to the fetus and extraembryonic mesoderm, while the ability to colonize trophectoderm is substantially restricted (Beddington, R.S.P. *et al., Development, 105*:733-738 (1989)). Thus, there is a high probability that embryonic stem cells are deficient in trophectodermal potential and mainly retain the capability of differentiating into embryonic lineages. If this is the case, then after transfer into an egg-cell cytoplasm, full trophectodermal potential should be regained. If this is not the case, then formation of a normal blastocyst and trophectoderm can be disturbed, affecting, or even precluding, post-implantation development.

Indeed, in certain cases, the reprogramming of embryonic stem cell nuclei occurs, since about 10% of the reconstituted embryos in this study were able to form blastocysts. Further, some were able to implant and a few developed to days 13-16.

Having now fully described this invention, it will be understood by those with skill in the art that the same may be performed within a wide and equivalent range of conditions, parameters, and the like, without affecting the spirit or scope of the invention or of any embodiment thereof.

What Is Claimed Is:

1. A method for cloning an animal comprising introducing embryonic stem cells from an animal species into a blastocyst of said animal species, from which blastocyst the inner cell mass has been removed.

2. A method for producing an embryo of an animal species comprising introducing embryonic stem cells from said animal species into a blastocyst of said animal species, from which blastocyst the inner cell mass has been removed.

3. The method of either of claims 1 or 2 wherein said animal or embryo is mammalian.

4. The method of claim 3 wherein said mammalian animal or embryo is bovine.

5. A method for cloning an animal comprising introducing an isolated nucleus from an embryonic stem cell from an animal species into an oocyte of said animal species, from which oocyte the nucleus has been removed.

6. A method for producing an embryo of an animal species comprising introducing a nucleus from an embryonic stem cell from said animal species into an oocyte of said animal species, from which oocyte the nucleus has been removed.

7. The method of either of claims 5 or 6 wherein said animal or embryo is mammalian.

8. The method of claim 7 wherein said mammalian animal or embryo is bovine.

9. A method for cloning an animal comprising introducing an isolated nucleus from an embryonic stem cell from an animal species into a two-celled embryo of said animal species, from which embryo the nuclei have been removed.

10. A method for producing an embryo of an animal species comprising introducing a nucleus from an embryonic stem cell from said animal species into a two-celled embryo of said animal species, from which embryo the nuclei have been removed.

11. The method of either of claims 9 or 10 wherein said animal or embryo is mammalian.

12. The method of claim 11 wherein said mammalian animal or embryo is bovine.

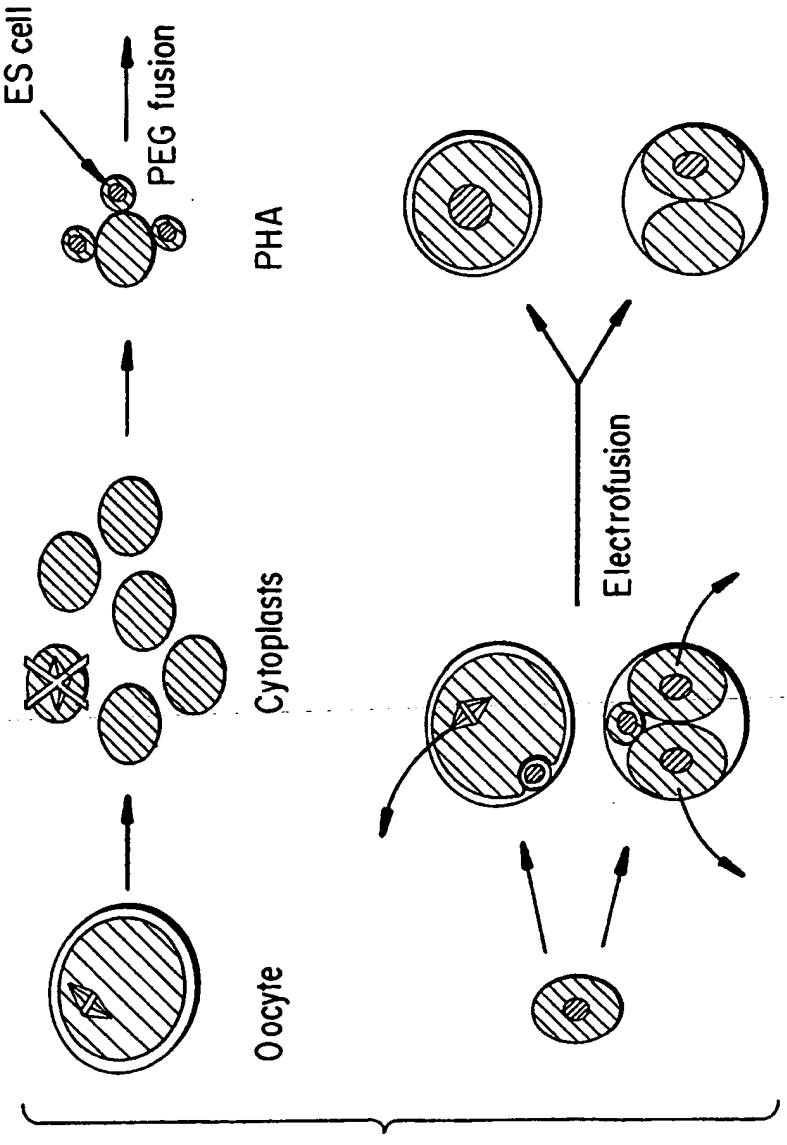

FIG. 1

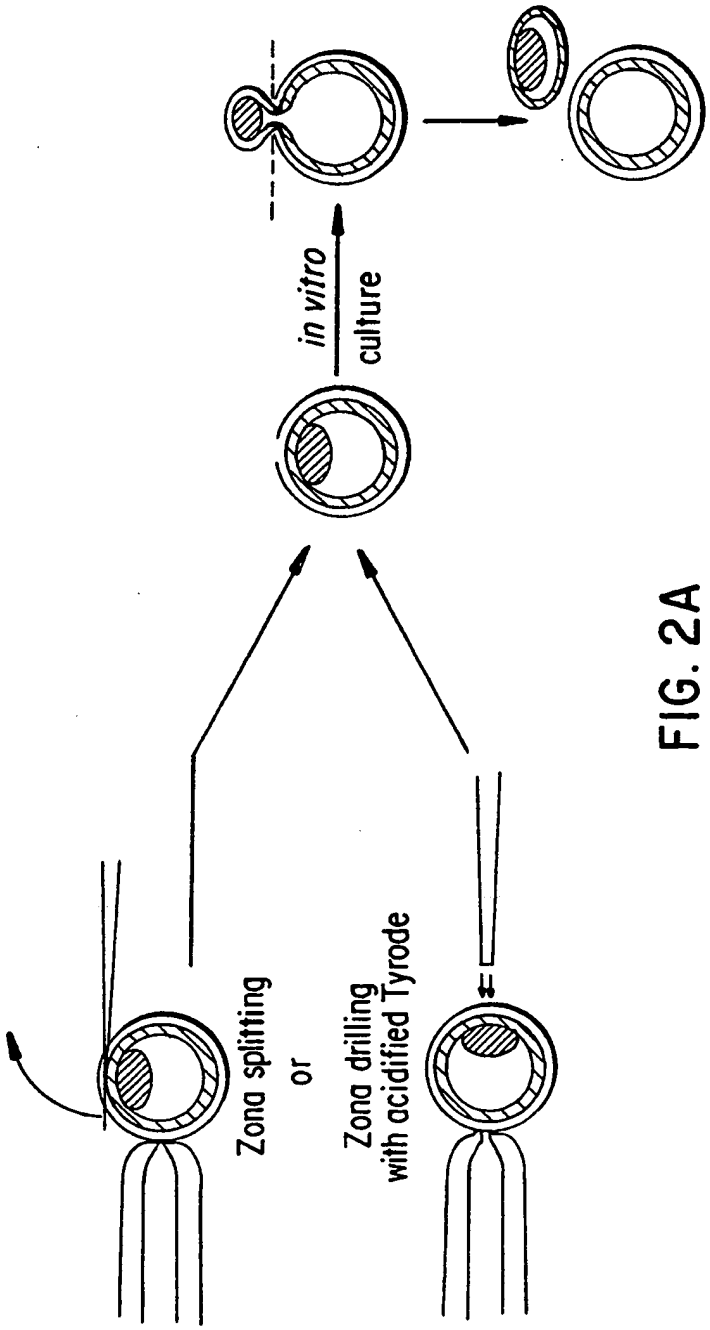
in vitro culture
Zona splitting
or
Zona drilling
with acidified Tyrode

FIG. 2A

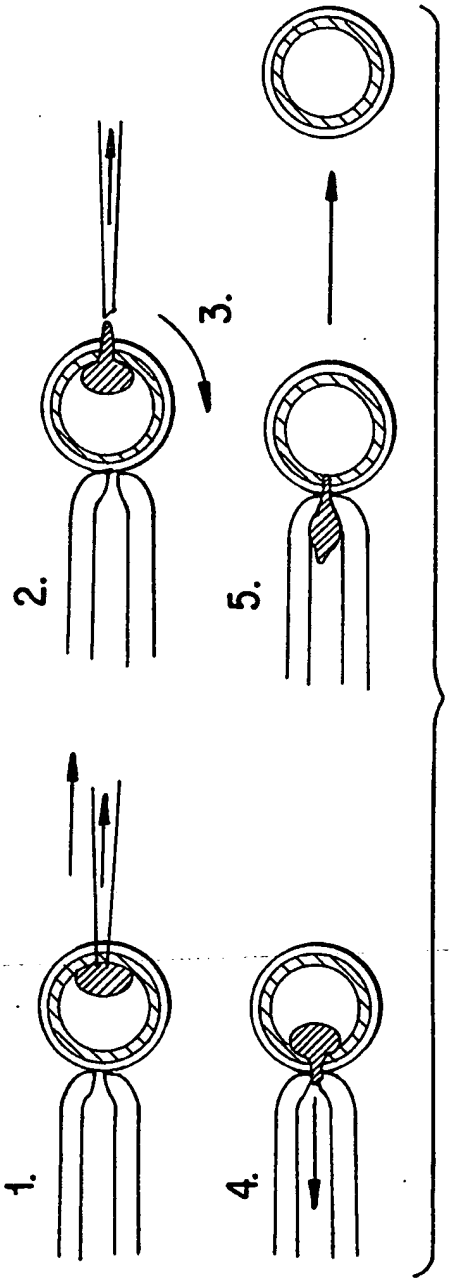

FIG. 2B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08878

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N-15/00, 15/06
US CL :435/172.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN-Medline
Search Terms: Modlinski, J?/au; Reed, M?/au; Wagner, T?/au; embyro?; nucle?; transplant?; stem; cell?; ablat?; remov?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DEVELOPMENT, Volume 110, issued 1990, J. Nichols et al., "Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity", pages 1341-1348, see entire document.	1-12
Y	JOURNAL OF EMBRYOLOGY AND EXPERIMENTAL MORPHOLOGY, Volume 87, issued 1985, T.C. Doetschman et al., "The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium", pages 27-45, see entire document.	1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 DECEMBER 1993	Date of mailing of the international search report 12 JAN 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer BRIAN R. STANTON Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08878

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EXPERIMENTAL CELL RESEARCH, Volume 190, issued 1990, S. Pease et al., "Formation of Germ-Line Chimeras from Embryonic Stem Cells Maintained with Recombinant Leukemia Inhibitory Factor", pages 209-211, see entire document.	1-12
Y	TRENDS IN GENETICS, Volume 5, number 8, issued August 1989, J. Rossant et al., "Towards a molecular genetic analysis of mammalian development", pages 277-283, see entire document.	1-12
Y	THE EMBO JOURNAL, Volume 9, number 10, issued 1990, E.F. Wagner, "On transferring genes into stem cells and mice", pages 3025-3032, see entire document.	1-12
Y	SCIENCE, Volume 240, issued 10 June 1988, R. Jaenisch, "Transgenic Animals", pages 1468-1474, see entire document.	1-12
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Y	JOURNAL OF EMBRYOLOGY AND EXPERIMENTAL MORPHOLOGY, Volume 73, issued 1983, M.H. Kaufman et al., "Establishment of pluripotential cell lines from haploid mouse embryos", pages 249-261, see entire document.	1-12
Y	TRENDS IN GENETICS, Volume 2, issued January 1986, E.J. Robertson, "Pluripotential stem cell lines as a route into the mouse germ line", pages 9-13, see entire document.	1-12
Y	BIOTECHNOLOGY, Volume 6, number 10, issued October 1988, J. Van Brunt, "Molecular Farming: Transgenic Animals as Bioreactors", pages 1149-1154, see entire document.	1-12
Y	JOURNAL OF CELL SCIENCE, SUPPLEMENT, Volume 10, issued 1988, J.K. Heath et al., "Regulatory Factors of embryonic stem cells", pages 257-266, see entire document.	1-12
Y	JOURNAL OF REPRODUCTION AND FERTILITY, Volume 93, issued 1991, T. Kono et al., "Development of enucleated mouse oocytes reconstituted with embryonic nuclei", pages 165-172, see entire document.	1-12
Y	NATURE, Volume 273, issued 08 June 1978, J.A. Modlinski, "Transfer of embryonic nuclei to fertilised mouse eggs and development of tetraploid blastocysts", pages 466-467, see entire document.	1-12

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PCT/US93/08878

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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, Volume 292, issued 23 July 1981, J.A. Modlinski, "The fate of inner cell mass and trophectoderm nuclei transplanted to fertilized mouse eggs", pages 342-343, see entire document.	1-12
Y	NATURE, Volume 320, issued 06 March 1986, S.M. Willadsen, "Nuclear transplantation in sheep embryos", pages 63-65, see entire document.	1-12
Y	SCIENCE, Volume 220, issued 1983, J. McGrath et al., "Nuclear Transplantation in the Mouse Embryo by Microsurgery and Cell Fusion", pages 1300-1302, see entire document.	1-12
Y	SCIENCE, Volume 226, issued 14 December 1984, J. McGrath et al., "Inability of Mouse Blastomere Nuclei Transferred to Enucleated Zygotes to Support Development in Vitro", pages 1317-1319, see entire document.	1-12
Y	JOURNAL OF CELL SCIENCE, Volume 69, issued 1984, R. Czolowska et al., "Behavior of thymocyte nuclei in non-activated and activated mouse oocytes", pages 19-34, see entire document.	1-12
Y	JOURNAL OF CELL SCIENCE, Volume 84, issued 1986, R.Czolowska et al., "Chromosome condensation activity in ovulated metaphase II mouse oocytes assayed by fusion with interphase blastomeres", pages 129-138, see entire document.	1-12
Y	CELL, Volume 23, issued January 1981, K. Illmensee, "Nuclear Transplantation in Mus musculus: Developmental Potential of Nuclei from Preimplantation Embryos", pages 9-18, see entire document.	1-12
Y	DEVELOPMENT, Volume 107, issued 1989, Y. Tsunoda et al., "Nuclear transplantation of male primordia germ cells in the mouse", pages 407-411, see entire document.	1-12
Y	DEVELOPMENT, Volume 101, issued 1987, S.K. Howlett et al., "Nuclear cytoplasmic interactions following nuclear transplantation in mouse embryos", pages 915-923, see entire document.	1-12
Y	DEVELOPMENT, Volume 108, issued 1990, J.A. Modlinski et al., "Nuclear transfer from teratocarcinoma cells into mouse oocytes and eggs", pages 337-348, see entire document.	1-12
Y	US, A, 5,057,420 (MASSEY) 15 OCTOBER 1991, see entire document.	1-12

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,873,191 (WAGNER ET AL) 10 OCTOBER 1989, see entire document.	1-12
Y	US, A, 4,619,899 (NIKITIN ET AL) 28 OCTOBER 1986, see entire document.	1-12
Y	US, A, 5,032,407 (WAGNER ET AL) 16 JULY 1991, see entire document.	1-12
Y	US, A, 4,806,476 (COONS ET AL) 21 FEBRUARY 1989, see entire document.	1-12
Y	US, A, 4,664,097 (MCGRATH) 12 MAY 1987, see entire document.	1-12

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)
Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-4, drawn to methods of cloning an animal by injection of blastocysts with embryonic stem cells, classified in Class 435, subclass 172.3.

II. Claims 5-8, drawn to second methods of cloning an animal by nuclear transplantation into an oocyte, classified in Class 435, subclass 172.3.

III. Claims 9-12, drawn to third methods of cloning an animal by nuclear transplantation into two celled embryos, classified in Class 435, subclass 172.3.

The inventions are distinct one from the other because they are drawn to materially different methods of animal cloning which require separate and distinct considerations as well as non-coextensive fields of search in the non-patent literature. The three different methods are predicated upon fundamentally different principles. In the first method, blastocystic support cells (trophectoderm) are used to promote the development of pluripotent embyronic stem cells. In the second two methods, acellular nuclei are transplanted into fundamentally different host cells. In the invention of group II, the host cell is a haploid oocyte whereas in the invention of group III the host cell is a diploid cell of an early embryo.

Since as noted above, the biology of the three groups of inventions is distinct, different considerations and areas of search are required. Therefore, the three groups of inventions are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. Note that PCT Rule 13 does not provide for multiple methods within a single application.